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(57) Abstract

A method for the detection of *Francisella tularensis* in clinical samples based on a nested polymerase chain reaction (PCR) for the *FopA* gene using primers selected from the nucleotide sequences FNA8L (sequence GAGGAGTCTCAATGTACTAAGGTTTGCCC), FNB2L (sequence CACCATTATCCTGGATATTACCAGTGTCAT), FNA7L (sequence CTTGAGTCTTATGTTTCGGCATGTGAATAG) and FNB1L (sequence CCAACTAATTGGTTGTACTGTACAGCGAAG).

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DETECTION OF FRANCISELLA TULARENSIS USING OLIGONUCLEOTIDE PROBES

Francisella tularensis is the causative agent of tularemia in man and animals. The species is divided into two subspecies: *Francisella tularensis* subspecies *tularensis* (type A) and *Francisella tularensis* subspecies *palaearctica* (type B). Type A strains (which are confined to Nearctic regions) cause a more serious disease with higher mortality than that caused by type B strains (which are of Holarctic distribution). The agent is extremely infectious with as few as ten organisms capable of causing disease in humans (See Bell J, 1983. Tularemia. In: Stoenner H, Kaplan W, Torten M, editors. Handbook series in zoonoses. Section A bacterial, rickettsial, and mycotic diseases. New York: Academic press, 161-193.

Current methods of diagnosis are unsatisfactory. The symptoms of tularemia are not pathognomic and the disease is often diagnosed as atypical pneumonia. Blood culture is slow (48 - 72) hours, prone to false negatives, and represents a considerable safety hazard to laboratory workers (Weaver RE, Hollis DG, Bottone EJ, 1985. Gram-negative fermentative bacteria and *Francisella tularensis*. In: Lennette EH, Balows A, Hausler WJ, Shodomy HJ, editors. Manual of clinical microbiology. 4th ed. Washington D.C. American society for microbiology, 309-329). Measurement of the increase in circulating antibody titre in convalescent serum provides important epidemilogical data. but is of little value in the management of the disease. Rapid diagnosis is particularly important for tularemia to select the appropriate antibiotic therapy. The organism is naturally resistant to penicillin, and treatment with antibiotics that cannot penetrate cells, such as gentamicin, fail to eliminate the bacteria and relapses of the disease occur (Enderlin G, Morales L, Jacobs RF, Cross JT, 1994. Streptomycin and alternative agents for the treatment of tularemia: review of the literature. *Clin Infect Dis 19:*42-47).

Because *Francisella tularensis* is highly infectious by the aerosol route and causes severe disease it is recognised as an important biological warfare agent (Spencer RC, Wilcox MH, 1993. Agents of biological warfare. *Rev Med Microbiol 4:*138-143.). Therefore there is a military requirement to rapidly identify the organism.

The gene encoding the protein that elicits the dominant antibody response, *FopA*, has been cloned into *Escherichia coli* and the nucleotide sequence determined (Leslie DL, Cox J, Lee M, Titball RW, 1993. Analysis of a cloned *Francisella tularensis* outer membrane protein gene and expression in attenuated Salmonella typhimurium. *FEMS MicrobiolLetts 111*:331-335.). Antibodies to the *FopA* are always present in human convalescent serum (Bevanger L, Maeland JA, Naess AI, 1989. Competitive enzyme immunoassay for antibodies to a 43,000-molecular-weight *Francisella tularensis* outer membrane protein for the diagnosis of tularemia. *J Clin Microbiol 27:922-926.*) and therefore it is well developed in the *Francisella* genera.

According to this invention a method for the detection of the bacterium *Francisella tularensis* comprises the steps of hybridizing one or more sequence of nucleic acids associated with the bacterium, detecting hybridization products and relating the detection of hybridization products to the presence of the bacterium, and is characterised in that the nucleic acid sequence or sequences hybridized is located in the *FopA* outer membrane protein gene and in that hybridization of one or more sequence of nucleic acids is carried out using one or more primers having a sequence selected from any consecutive 10 or more bases from one or more of the nucleotide sequences:

GAGGAGTCTCAATGTACTAAGGTTTGCCC	FNA8L
CACCATTATCCTGGATATTACCAGTGTCAT	FNB2L
CTTGAGTCTTATGTTTCGGCATGTGAATAG	FNA7L
CCAACTAATTGGTTGTACTGTACAGCGAAG	FNB1L

A preferred embodiment further includes the step of amplifying one or more sequence of nucleic acids associated with the bacterium.

In a further preferred embodiment the amplification of one or more sequence is achieved by a polymerase chain reaction.

In a further preferred embodiment the amplification of one or more sequence is achieved by a nested polymerase chain reaction.

The invention will now be described, by way of illustration. with reference to non-limiting examples and the following figures in which figure 1 shows the sensitivity of the technique in detecting purified *Francisella tularensis* DNA in the presence of murine blood and figure 2 shows the sensitivity of the technique in detecting purified *Francisella tularensis* DNA in infected murine blood. Further embodiments will occur to those skilled in the art in light of these.

Bacterial Stains and Culture. *Francisella tularensis* was grown on blood cysteine glucose agar and modified cysteine partial hydrolysate broth (MCPH). *Francisella tularensis* used for animal challenge was washed three times in phosphate buffered saline (PBS), re-suspended in MCPH broth supplemented with 10% glycerol, and stored in 0.5ml volumes at -70°C.

Preparation of amplifiable DNA from clinical samples.

Method 1: Spleens were homogenised in 10ml PBS in a Stomacher Lab-Blender Model 80 (Seward Medical, London) for 2 min, the homogenate diluted 1/10, and Gene Releaser added 1:1. 10µl were subjected to the manufacturers recommended thermal cycle before a standard PCR was completed.

Method 2 (Based on Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim van Dillen PM, van der Noordaa J, 1990. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol 28:*495-503.): Organs were homogenised in 2ml L6 buffer (8M guaninidium thyiocyanate, in 0.1M tris-HCl, pH 6.4 containing 44mM EDTA and 2.6% (v/v) Triton X-100). 100 µl of homogenate were added to a reaction vessel containing 900 µl L6 buffer and 40 µl acidified silica diatoms (Jansen Chimica).

The silica diatoms were washed twice with L2 buffer (8M guaninidium thiocyanate, in 0.1M tris-HCl, pH6.4), twice with ethanol, and once with acetone. The pellets were dried at 56°C, 100µl distilled water added, and the DNA was eluted from the silica at 56°C for 5 minutes. The reaction vessels were centrifuged and the DNA removed and stored at -70°C. To determine the relative sensitivity of the two methods spleen and blood were spiked with various numbers of *Francisella tularensis* before processing and subjected to PCR.

Challenge. Mice were challenged with a 5 x 10^5 CFU of *Francisella tularensis* Live Vaccine Strain suspended in PBS intraperitoneally. At 24 hour intervals mice were anaesthetized by halothane and blood removed by cardiac puncture. Mice were immediately killed by cervical dislocation and spleens removed. 50µl of blood were plated on to blood cysteine glucose agar.

PCR. PCR was performed using a Perkin-Elmer PE9600 thermocycler. The thermal cycle was 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds. A final elongation of 72°C for 10 minutes was followed by storage at 4°C until analysis. For each reaction 20µl of a reaction mixture (1.5µM0 primers, 300µM each dNTP, 4.125mM $MgCl_2$ and 0.75 U Boehringer Mannheim *Taq* polymerase in 1.5X Boehringer Mannheim reaction buffer) were added to 10µl DNA sample. For some experiments cosolvents DMSO, foramide or glycerol were added to the PCR master mix (see Rolfs A, Schuller I, Finckh U, Weber-Rolfs I, 1992. Substances affecting PCR: Inhibition or enhancement. In: Anonymous PCR: Clinical diagnostics and research. Berlin: Springer-Verlag, 51-60.). For the first PCR, the following primer pairs were used FNA8L (sequence GAGGAGTCTCAATGTACTAAGGTTTGCCC) and FNB2L (sequence CACCATTATCCTGGATATTACCAGTGTCAT). The inner (nested pair) primers were FNA7L (sequence CTTGAGTCTTATGTTTCGGCATGTGAATAG) and FNB1L sequence CCAACTAATTGGTTGTACTGTACAGCGAAG).

Detection of PCR Products. PCR products were detected by UV induced fluorescence following electrophoresis through 2% aggarose abd staining by ethidium bromide (see Sambrook J, Fritsch EF, Maniatis T, Molecular cloning, A Laboratory Manual, 1989, 2nd ed. Cold Spring Harbour, N.Y. Cold Spring Harbour Laboratory Press.).

Specificity of Primers. All primer pairs were tested against a panel of bacterial DNA comprising:-

Clostridium perfringens type A strain NCTC 8237, type B strain NCTC 8533, type C strain NCTC 10719, type D strain NCTC 8346;
Clostridium botulinim undesignated type E strain, undesignated type F strain;
Bacillus subtilis NCTC 10412;
Bacillus cereus strain NCTC 9939;
Bacillus anthracis strain Ames;
Vibrio cholera strain NCTC 8029, strain NCTC 10782;
E. coli K12 strain JM101, strain 08: K87: K88ad:H19;
Psuedomonas aeruginosa strain 606;
Psuedomonas mallei strain NCTC 10230;
Psuedomonas psuedomallei strain 4845;
Yersinia pestis strain EV76;
Francisella tularensis strain Schu 4;
Francisella novicida strain ATCC 15482
and murine DNA.

Specificity of PCR assays. Attempts were made to amplify the *FopA* targeted sequences from 1ng samples of DNA isolated from *Francisella tularensis* LVS and HN63 and the strains in the bacterial DNA test panel listed in Materials and methods. PCR products of the appropriate size were detected from the *Francisella* derived DNA but not from any of the DNA isolated from the control bacteria. No detectable PCR product resulted from murine DNA.

Optimization of PCR. The PCR was optimized for a maximum product yield by altering the magnesium ion concentration and the annealing temperature in reactions using purified *Francisella tularensis* as template. The optimal annealing temperature was 55°C (range tested 45°C to 65°C) and the optimal magnesium ion final concentration was 4.25mM (range tested 1.5mM to 5.5mM).

Sensitivity of PCR Using Pure Culture. Using limiting dilution of viable *Francisella tularensis* LVS the respective sensitivities of standard and nested PCR were determined. It was found that standard PCR could detect approximately 500 CFU per reaction (equivalent to 5×10^4 CFU/ml) whereas nested PCR detected 1 CFU per reaction (equivalent to 100 CFU/ml).

Sensitivity of PCR from Clinical Samples. Blood and spleen homogenate were spiked with various numbers of *Francisella tularensis* and processed using both methods 1 and 2 described above. Method 1 required a 10 fold dilution to eliminate PCR inhibition. This resulted in a detection limit of 3×10^3 CFU per reaction (equivalent to a total bacterial count in the spleen of 3×10^6 CFU). Published data indicates that the total bacterial count in tularemic spleens is frequently lower than 3×10^6 CFU (see Sjostedt A, Sandstrom G, Tarnvik A, 1992. Humoral and cell-mediated immunity in mice to a 17-kilodalton lipoprotein of *Francisella tularensis* expressed by *Salmonella typhimurium. Infect and Immun 60*:2855-2862.) and so an alternative method was sought.

Method 2 had a detection limit of 5×10^2 CFU per reaction (equivalent to 5×10^4 CFU spleen total bacterial count). Attempts to improve the detection limit by using cosolvents (glycerol, DMSO and foramide: see Rolfs A, Schuller I, Finckh U, Weber-Rolfs I, 1992. Substances affecting PCR: Inhibition or enhancement. In: Anonymous PCR: Clinical diagnostics and research. Berlin: Springer-Verlag, 51-60) were not successful. Referring to figure 1 *Francisella tularensis* in ten fold dilutions were added to murine blood and the DNA extracted according to method 2. The DNA was amplified using either standard PCR (lanes A-C) or nested PCR (lanes E-G). Lanes D and H are the respective negative controls. The results showed that detection was further enhanced by using a nested PCR (1 CFU/reaction - equivalent to 1×10^2 CFU/ml).

Detection of *Francisella tularensis* from Infected Tissues. Nine mice were infected with *Francisella tularensis* LVS. At 24 hour intervals three mice were killed, their spleens removed and blood samples taken. The infection dose was selected to cause death after 3-4 days. Using method 2 followed by a standard PCR, *Francisella tularensis* DNA could be detected in five spleens and three blood samples at the height of tularemic infection.

However, using nested PCR, *Francisella tularensis* could be detected in all three spleens samples and in eight (out of nine) blood samples. The absence of bacteria in the one PCR negative sample was confirmed by blood culturing. Referring to figure 2, lanes B-D relate to 24 hour samples lanes E-G relate to 48 hour samples and lanes H-J relate to 72 hours. Lanes A and K are negative controls. The lowest positive blood culture was 1 CFU/µl. Thus the development of a nested PCR based test has significantly increased the sensitivity previously reported (see Long GW, Oprandy JJ, Narayanan RB, Fortier AH, Porter KR, Nacy CA, 1993. Detection of *Francisella tularensis* in blood by polymerase chain reaction. *J Clin Microbiol 31:*152-154.

Further reductions in the time taken to complete diagnosis are envisaged as PCR technology develops. For example, thermocyclers that can use capillary tubes can significantly reduce the time for this process. Also capillary electrophoresis reduces the time taken to analyse PCR products.

Claims.

1. A method for the detection of the bacterium *Francisella tularensis* comprising the steps of hybridizing one or more sequence of nucleic acids associated with the bacterium, detecting hybridization products and relating the detection of hybridization products to the presence of the bacterium, characterised in that the nucleic acid sequence or sequences hybridized is located in the *FopA* outer membrane protein gene and in that hybridization of one or more sequence of nucleic acids is carried out using one or more primers having a sequence selected from any consecutive 10 or more bases from one or more of the nucleotide sequences:

GAGGAGTCTCAATGTACTAAGGTTTGCCC	FNA8L
CACCATTATCCTGGATATTACCAGTGTCAT	FNB2L
CTTGAGTCTTATGTTTCGGCATGTGAATAG	FNA7L
CCAACTAATTGGTTGTACTGTACAGCGAAG	FNB1L

2. The method of claim 1 and further including the step of amplifying one or more sequence of nucleic acids associated with the bacterium.

3. The method of claim 2 where the amplification of one or more sequence is achieved by a polymerase chain reaction.

4. The method of claim 3 where the amplification of one or more sequence is achieved by a nested polymerase chain reaction.

5. An oligonucleotide hybridization probe, transcription primer or specific sequence amplification primer comprising a sequence selected from any 10 or more consecutive bases selected from any of the nucleotide sequences:

GAGGAGTCTCAATGTACTAAGGTTTGCCC	FNA8L
CACCATTATCCTGGATATTACCAGTGTCAT	FNB2L
CTTGAGTCTTATGTTTCGGCATGTGAATAG	FNA7L
CCAACTAATTGGTTGTACTGTACAGCGAAG	FNB1L

Fig.1.

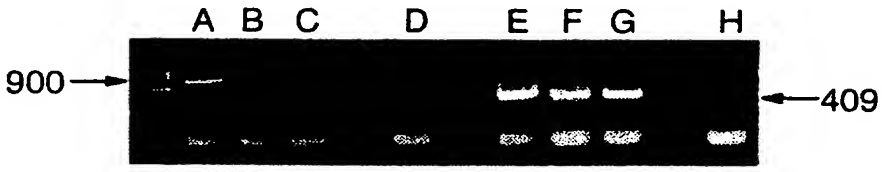
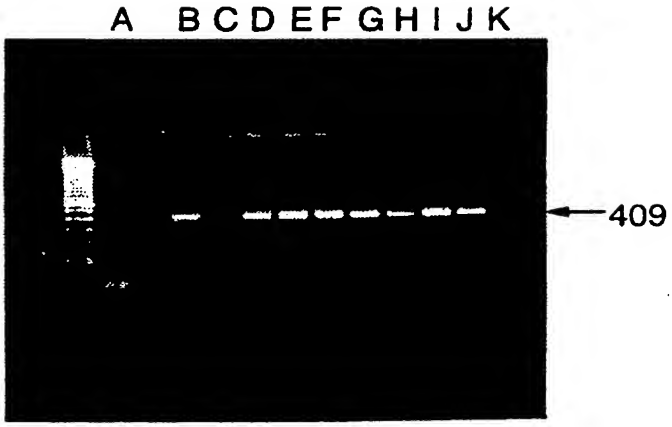


Fig.2.



INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEMS MICROBIOLOGY LETTERS, vol. 111, 1 August 1993, pages 331-5, XP000614904 LESLIE D ET AL: "Analysis of a cloned francisella tularensis outer membrane protein gene and expression in attenuated Salmonella typhimurium" see page 334, paragraph 3 ---	1-5
X	JOURNAL OF CLINICAL MICROBIOLOGY, vol. 31, no. 1, January 1993, pages 152-4, XP000614920 LONG G ET AL: "Detection of Francisella tularensis in blood by polymerase chain reaction" see the whole document --- -/--	1-5

[X] Further documents are listed in the continuation of box C.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AMERICAN JOURNAL TROPICAL MEDICINE AND HYGIENE. , XP000614907 LONG G ET AL: "Polymerase chain reaction assay for detection of Francisella tularensis" see abstract 42 see page 110	1-5
A	DATABASE WPI Week 9230 Derwent Publications Ltd., London, GB; AN 92-247974 XP002024048 "E.coli strain contg. recombinant DNA pR D6 - is used to produce probe for testing Francisella tularensis bacteria" & SU,A,1 669 981 (ROST ANTIPLAGUE RES INST) , 15 August 1991 see abstract	1-4
A	BIOTEKHNOLOGIYA, vol. 4, 1992, pages 76-81, XP000614952 ROMANOVA L ET AL: "DNA probe for identifying bacteria of the genus Francisella" see the whole document	1-4
X	EP,A,0 522 880 (INTERNATIONAL FLOWER DEVELOPMENTS PTY. LTD.) 13 January 1993 see page 15, line 28	5

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INTERNATIONAL SEARCH REPORT

Information on patent family members

In[illegible]tional Application No
PCT/GB 96/02262

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-522880	13-01-93	WO-A- 9301290	21-01-93
		CA-A- 2112373	21-01-93
		JP-T- 6500239	13-01-94
		NZ-A- 243500	27-06-94
		PL-A- 298239	07-03-94
		US-A- 5569832	29-10-96
		US-A- 5349125	20-09-94